

VU Research Portal

X-linked creatine transporter deficiency

van de Kamp, J.M.

2014

document version

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

citation for published version (APA)

van de Kamp, J. M. (2014). *X-linked creatine transporter deficiency*. [, Vrije Universiteit Amsterdam].

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:

vuresearchportal.ub@vu.nl



**Creatine transporter deficiency in
male patients**

2

Phenotype and genotype in 101 males with X-linked creatine transporter deficiency



van de Kamp JM*, Betsalel OT*, Mercimek-Mahmutoglu S, Abulhoul L, Grünewald S, Anselm I, Azzouz H, Bratkovic D, de Brouwer A, Hamel B, Kleefstra T, Yntema H, Campistol J, Vilaseca MA, Cheillan D, D'Hooghe M, Diogo L, Garcia P, Valongo C, Fonseca M, Frints S, Wilcken B, von der Haar S, Meijers-Heijboer EJ, Hofstede F, Johnson D, Kant SG, Lion-Francois L, Pitelet G, Longo N, Maat-Kievit JA, Monteiro JP, Munnich A, Muntau AC, Nassogne MC, Osaka H, Ounap K, Pinard JM, Quijano-Roy S, Poggenburg I, Poplawski N, Abdul-Rahman O, Ribes A, Arias A, Yaplito-Lee J, Schulze A, Schwartz CE, Schwenger S, Soares G, Sznajer Y, Valayannopoulos V, Van Esch H, Waltz S, Wamelink MMC, Pouwels PJW, Errami A, van der Knaap MS, Jakobs C, Mancini GM, Salomons GS

ABSTRACT

Background

Creatine transporter deficiency is a monogenic cause of X-linked intellectual disability. Since its first description in 2001 several case reports have been published but an overview of phenotype, genotype and phenotype-genotype correlation has been lacking.

Methods

We performed a retrospective study of clinical, biochemical and molecular genetic data of 101 males with X-linked creatine transporter deficiency from 85 families with a pathogenic mutation in the creatine transporter gene (*SLC6A8*).

Results and conclusions

Most patients developed moderate to severe intellectual disability; mild intellectual disability was rare in adult patients. Speech language development was especially delayed but almost a third of the patients were able to speak in sentences. Besides behavioral problems and seizures, mild to moderate motor dysfunction, including extrapyramidal movement abnormalities, and gastrointestinal problems were frequent clinical features. Urinary creatine/creatinine ratio proved to be a reliable screening method besides MR spectroscopy, molecular genetic testing, and creatine uptake studies, allowing definition of diagnostic guidelines. A third of patients had a de novo mutation in the *SLC6A8* gene. Mothers with an affected son with a de novo mutation should be counseled about a recurrence risk in further pregnancies due to the possibility of low level somatic or germline mosaicism. Missense mutations with residual activity might be associated with a milder phenotype and large deletions extending beyond the 3' end of the *SLC6A8* gene with a more severe phenotype. Evaluation of the biochemical phenotype revealed unexpected high creatine levels in cerebrospinal fluid suggesting that the brain is able to synthesize creatine and that the cerebral creatine deficiency is caused by a defect in the reuptake of creatine within the neurons.

INTRODUCTION

In 2001, a 6 year-old boy with intellectual disability (ID), severe speech delay, mild hypotonia, short attention span, and status epilepticus was evaluated. The severely reduced cerebral creatine signal on proton MR spectroscopy (¹H-MRS) in combination with a family history suspect for X-linked inheritance and an increased creatine/creatinine (Cr/Crn) ratio in urine led to the discovery of the X-linked creatine transporter deficiency (CRTR-D).¹

² Creatine uptake in cultured skin fibroblasts was deficient and a hemizygous nonsense mutation was detected in the *SLC6A8* gene, which is located on the X-chromosome.

CRTR-D comprises together with the autosomal recessive creatine biosynthesis defects arginine:glycine amidinotransferase (AGAT) deficiency and guanidinoacetate methyltransferase (GAMT) deficiency, the cerebral creatine deficiency syndromes.

Shortly after the description of the first patient, more patients were diagnosed and the prevalence of the disorder was estimated between 0.3% and 3.5% in males with ID³⁻⁷ and around 2% in X-linked-ID.³⁻⁷ Several case-reports have been published,^{6-8, 8-36} however, an overview has been lacking.

Creatine is mainly known for its essential role in energy metabolism as the creatine/phosphocreatine system serves as a buffer in the regeneration of ATP and as a shuttle of high-energy phosphates between mitochondrial sites of production to cytosolic sites of utilization.³⁷ However, creatine might also have a neuromodulatory role.³⁸ Creatine is derived from the diet and endogenous synthesis from arginine and glycine. Originally, it was thought that the first step of creatine synthesis (catalyzed by AGAT), yielding guanidinoacetate (GAA), took place mainly in the kidney and the second step (catalyzed by GAMT) in the liver and that creatine was taken from the blood by creatine-requiring tissues. However, the expression of AGAT and GAMT enzymes seems to be more widespread and the contribution of various tissues to creatine synthesis is still unclear.³⁷

Here we present an overview of the clinical spectrum of the CRTR-D with clinical, biochemical and molecular genetic data of a large cohort including 101 males from 85 families. This overview provides directions for the management of patients and counselling of families regarding prognosis and recurrence risks. Diagnostic guidelines are proposed and possible genotype-phenotype relations are discussed. The study also reveals important clues about the disease patho-mechanism and creatine metabolism.

METHODS

Collection of clinical and biochemical data

The VU University Medical Centre (VUMC), Amsterdam was the first centre performing molecular genetic analysis of the *SLC6A8* gene. Until October 2012, a pathogenic mutation had been detected in 109 male probands from all over the world. Comprehensive questionnaires were sent to the physicians of males with a pathogenic mutation in the *SLC6A8* gene. Additionally, all male patients published up to October 2012 as case reports were included. Female heterozygotes were not included in this study.

The study was approved by the ethics committee of the VUMC, Amsterdam, The Netherlands.

Analysis of the *SLC6A8* gene

All 13 exons of the *SLC6A8* gene (NM_005629.3) were sequenced as previously described³⁹ at the VUMC in 87 of the 103 patients in this cohort. DNA analysis in the other 16 patients was performed in various other laboratories.

In four cases a large deletion was detected. This was confirmed by multiplex ligation-dependent probe amplification (MLPA) using the P049 kit according to standard protocol (MRC-Holland, The Netherlands). The breakpoints were amplified by long range PCR and confirmed by direct DNA sequence analysis.

Novel missense variants were further characterized by testing for restoration of creatine uptake in CRTR deficient fibroblasts after transfection with *SLC6A8* cDNA in which the variant had been introduced by site-directed mutagenesis.^{40, 41} Novel neutral or intronic variants were further characterized by bioinformatics analysis and mRNA analysis.³⁹

Creatine uptake in skin fibroblasts

The creatine uptake after incubation at 25 μ M creatine was measured as previously described.⁴¹

Statistical analysis genotype-phenotype correlation

An unpaired t-test was applied for statistical analysis.

RESULTS

Patients

Questionnaires of 77 males from 64 families were received. A total of 30 of these 77 patients were also reported in previous case reports or treatment trials.^{7, 10, 12, 13, 15, 16, 20-23, 26, 27, 29, 31, 42} Two patients were excluded because of the presence of a large deletion including neighbouring gene(s).^{12, 31} Additionally 26 previously reported males from 23 families^{1, 2, 6-9, 11, 12, 14, 17-19, 22, 24, 26, 28, 30, 32-36} were included. In total 101 males from 85 families were included. Age varied between 1 and 66 years, with median age of 10 years (n=97). Twenty-one patients were adults (>18 years).

Phenotype

Presentation

The patients came to medical attention between birth and 6 years (n=82; mean 1 year). The presenting symptoms are shown in table 1.

Table 1. Symptoms at first presentation (n=87).

Sign	Present (%)
Developmental delay	80
Hypotonia	10
Gastrointestinal	16
Failure to thrive/growth delay/poor weight gain	8
Vomiting	5
Gastrointestinal reflux	2
Feeding difficulties/poor suck and swallowing	5
Seizures	10
Behavioral problems	6
Other (clubfoot, strabismus, adducted thumbs and laryngomalacia, PVCs, apnoea)	6

PVCs, premature ventricular contractions.

Development delay

Patients achieved independent walking at a mean age of 2 years (n=67; range 13 months-4 years). Speech development was delayed in all. First words were at a mean age of 3.1 years (n=44; range 9 months-10 years). The level of speech according to age is shown in Figure 1.

ID was classified as severe (IQ 20-34), moderate (IQ 35-49) or mild (IQ 50-69) based on neuropsychological testing if performed (n=48) or as estimated by the referring physician (n=43). The ID was moderate to severe in adult patients (Figure 2), only one 22-year-old male had mild ID (IQ 69).²⁰ One adult had progressive cognitive dysfunction.¹⁵

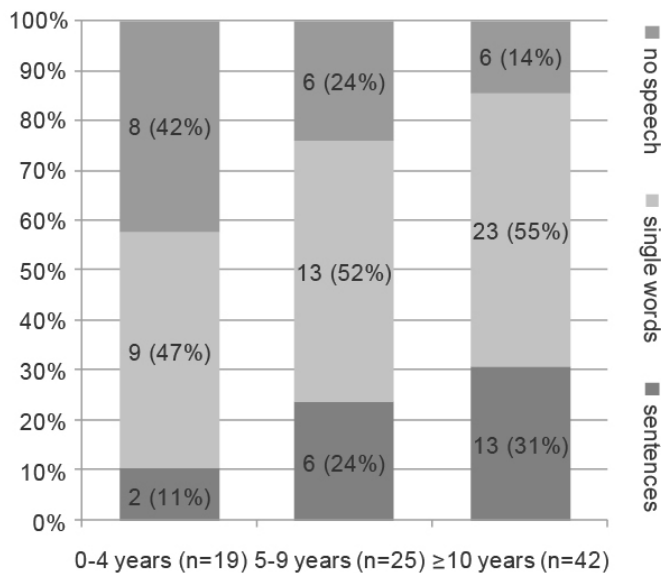


Figure 1. Level of speech according to age groups. In the columns the number of patients and the % of total number of patients are shown.

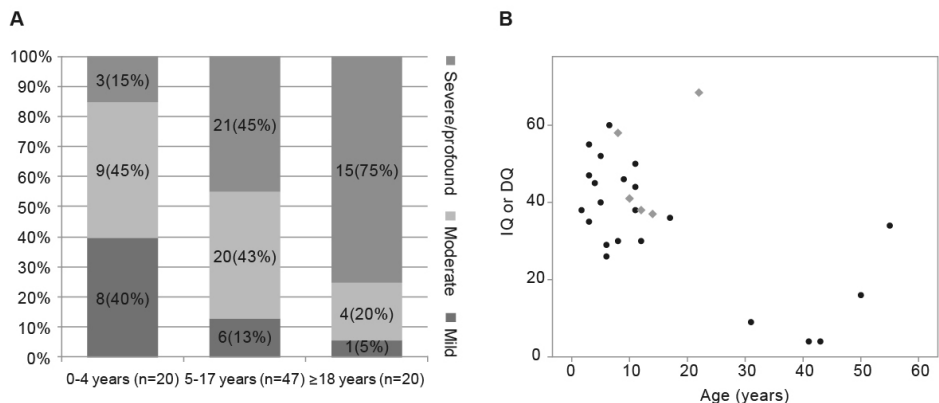


Figure 2. Degree of intellectual disability according to age groups (a) and Developmental Quotient (DQ) or Intelligence Quotient (IQ) scores for age (b). (a) In the columns the number of patients and the % of total number of patients are shown. (b) Exact DQ or IQ scores with ages were known in 27 patients (if more than one evaluation was known, the last was used). The gray diamonds depict patients with a missense mutation with residual activity. It should be noted that the four lowest IQs (scores 4-16) in the adults were brothers.¹⁰

Behavior problems

These were mentioned in 85% of patients. Attention deficit and/or hyperactivity (55%) and autistic features (41%) were the most common behavior problems followed by social anxiety/shyness (20%), stereotypic behavior (20%), impulsive behavior (27%), aggressive behavior (19%), self-injurious behavior (10%), and obsessive compulsive behavior (8%).

Seizures

Seizures were present in 59% of patients. Seizures were infrequent and well controlled with antiepileptic medication in most patients. The most common seizure types were generalized tonic-clonic seizure and simple or complex partial seizures with or without secondary generalization. Absence or myoclonic seizures occurred in a few patients. Patients often had febrile seizures in addition to non-febrile seizures. Three patients had severe refractory epilepsy. Status epilepticus occurred in nine patients. Mean age of onset for non-febrile seizures was 4.5 years (n=41; range 1-21 years).

Other neurological symptoms

Motor dysfunction, mostly mild to moderate, was mentioned in 58%. Hypotonia was most common (40%) and often improved with age. Other findings were signs of spasticity (stiff gait, mildly increased tonus, mildly increased reflexes) in 26%, coordination dysfunction (wide based/unstable gait, dysarthria, ataxia, clumsiness) in 29% and dystonia or athetosis (abnormal hand movements, intermittent dystonic posturing of the hands/wrists during walking, choreathetoid movements, dystonia of face and upper limbs) in 11%.

Additional symptoms

Gastrointestinal symptoms were reported in 35% and consisted of neonatal feeding difficulties (13%), failure to thrive (16%), vomiting (11%), (severe) chronic constipation (13%, nine out of 13 were adults), ileus (3%), hepatitis (n=1), gastric and duodenal ulcers (n=2), and hiatal hernia (n=1).

Urogenital anomalies were reported in 9% and consisted of dysfunctional voiding (3%), bladder instability, recurrent urinary infections, urethra stenosis, bilateral renal cysts, and unilateral testis hypoplasia (all n=1).

Ten patients (10%) had ophthalmologic abnormalities including strabismus (7%), bilateral abducens nerve palsy or Duane anomaly (n=2), mild cerebral visual deficit, and probably traumatic unilateral cataract and blindness (both n=1).

Four patients had mild (sensorial-neural) hearing loss.

Two previously described patients had mild cardiomyopathy.²⁰ One patient had multiple premature ventricular contractions.³³ One patient had long QT syndrome (QT time 495ms, reference 350-440).

Survival

Three patients, who were brothers, died: one at 21 years from tuberculosis and two around 40 and 60 years of unknown cause.

Family history

The family history was positive for X-linked intellectual disability (defined as ID in male relatives in the maternal line) in 20/85 (24%) index patients of which 18 had one or more (half-) brother(s) with ID. Five index patients had one or more (half-) sister(s) with ID. The mothers of 18/85 (21%) index patients had learning difficulties/mild ID of which 14 were proven heterozygotes and two had two or more affected sons but were not confirmed heterozygotes since no DNA analysis was performed.

Physical examination

Most patients had below average height while head circumference varied between +2 and -2 SD (Supplementary figure). Variable dysmorphic features were mentioned in 45% of the patients including broad/prominent forehead, midface hypoplasia, myopathic facies, ptosis, short nose, simple/unfolded/large ears, and joint laxity. Slender build and/or poorly developed muscular mass were frequent but not consistent signs.

Neuroimaging and MRS

Brain magnetic resonance imaging (MRI) was available in 76 patients and showed (mild) abnormalities in 53 patients, including mildly delayed myelination, (T2-) hyperintensities, thin corpus callosum, mildly enlarged ventricles/extracerebral spaces, and cerebral/cerebellar atrophy. Cerebral atrophy was progressive in two brothers.⁸

MRS results were available in 66 patients showing total absence or severe reduction of creatine.

Biochemical analysis

The quantitative urinary Cr/Crn ratio was available in 81 patients and was elevated in all (Figure 3). Additionally, six patients were said to have elevated levels.

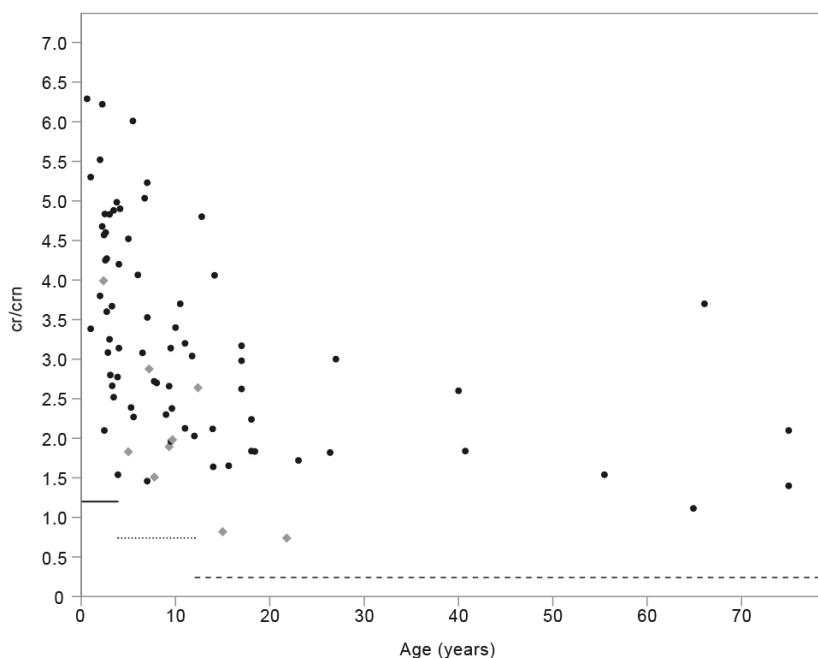


Figure 3. Urinary creatine/creatinine ratio shown for age in 81 patients (if more than one measurement was known, the lowest value was depicted). The *gray diamonds* depict patients with a missense mutation with residual activity. Upper limit of normal range for age < 4 years (1.2) is depicted with *uninterrupted line*, for age 4-12 years (0.74) with *small dashes* and for age > 12 years (0.24) with *large dashes*.

Creatine in plasma was available in 28 patients: 21 patients <10 years with normal levels between 67-105 μM , mean 90 μM (reference 17-109 μM^{43}) and seven patients >10 years with elevated levels between 60-103 μM , mean 82 μM (reference 6-50 μM^{43}).

Creatine in cerebrospinal fluid (CSF), available in seven untreated patients, varied between 51-80 μM (mean 67 μM) and was elevated in four (reference range 24-66 μM^{43}). Additionally, one patient had normal CSF creatine (62 μM) during creatine supplementation.

GAA in CSF, available in six untreated patients, was normal or slightly elevated (0.05-0.44 μM , mean 0.18 μM ; reference range 0.036-0.22 μM^{43}).

Creatinine in CSF was available in three (12-15 μM , mean 13 μM) and decreased in all (reference range 29-41 μM^{11}). Mildly or transient plasma lactate elevations and increased urinary 3-methylglutaconic acid and/or ethylmalonic acid and tricarboxylic acid cycle intermediates were mentioned in two patients with normal respiratory chain complex activities in a muscle biopsy.^{12, 27}

Creatine uptake in fibroblasts

We performed creatine uptake studies in cultured skin fibroblast in 41 patients and all had deficient uptake. Uptake expressed as per cent of uptake in control cell line used in the same test varied between 0-10.5% (mean 3.6%). Additionally, six patients were reported to have deficient uptake studies.

Diagnosis

Patients were detected by urinary, MRS or DNA investigations (Table 2). The diagnosis was confirmed by DNA analysis of the *SLC6A8* gene in all patients.

Table 2. Screening methods: initial diagnostic tests leading to the diagnosis in the 101 patients.

Initial test	%
Urinary Cr/Crn	39
MRS	21
Urinary Cr/Crn and MRS	4
DNA testing familial mutation	14
DNA sequencing*	10
Unknown	12

*performed in research cohorts of (X-linked) intellectual disability (n=9) or because of suspicion of CRTR-D based on clinical features (n=1).

Cr/Crn, creatine/creatinine ratio; CRTR-D, creatine transporter deficiency; MRS, MR spectroscopy.

Treatment

Twenty-one patients were included in previously published trials with L-arginine, alone or in combination with creatine and/or glycine. No substantial clinical effect or increase in cerebral creatine was seen in most patients^{21, 42, 44} although improvements were reported in single cases.^{34, 35} In addition, 26 patients were treated with either creatine monotherapy (n=5), a protocol including L-arginine (n=12) or an unspecified protocol (n=8). Data are lacking to assess the effectiveness in these patients.

Genotype

The most common mutation types were missense mutations (23 different mutations in 26/85 (31%) families) and one-amino acid (3 bp) deletions (nine mutations in 20/85 (24%) families). Most missense and 3bp deletions occurred in TM7 and TM8 (Figure 4). The

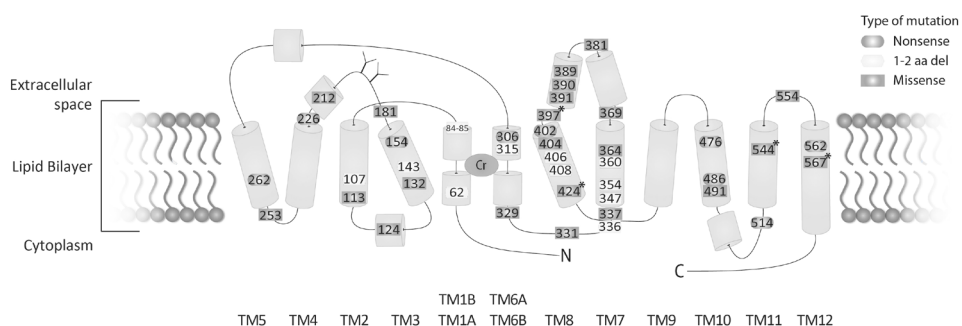


Figure 4. Locations of missense and 3bp deletions in *SLC6A8* protein. Missense mutations with a residual activity are marked by an asterisk (*).

pathogenicity of 20 out of the 23 missense mutations was confirmed by overexpression studies in creatine transporter deficient fibroblasts (Supplementary table). Notably four missense mutations, c.1271G>A; p.(Gly424Asp), c.1661C>T; p.(Pro544Leu), c.1699T>C; p.(Ser567Pro), and c.1190C>T; p.(Pro397Leu) had residual creatine transporter activity.

Less common mutation types were frameshift mutations (13 mutations in 15 families), nonsense mutations (nine mutations in eleven families), splice error mutations (intronic or synonymous variants with aberrant splicing by mRNA analysis and/or splice-site analysis tools) (seven mutations in nine families), and a translation initiation site mutation, c.1A>G; p.(Met1?) (one family).

In four unrelated patients, large multiple-exon deletions were detected, including two previously reported patients¹² of whom the breakpoints were now clarified. Two deletions extended in neighbouring gene(s) and these patients were excluded from this study. The other two patients had deletions of exons 5-12 and exons 8-13, respectively.

In total, 65 different mutations (Supplementary table) were identified. Ten mutations reoccurred in two or more families. The two most common mutations were c.1222_1224del; p.(Phe408del) detected in seven and c.1006_1008del; p.(Asn336del) in five unrelated families. All mutations are included in the Leiden Open Variation Database (LOVD) (<http://www.LOVD.nl/SLC6A8>).

Molecular genetic studies were performed in 61 mothers and the mutation was *de novo* in 18 (30%) sons. A low level mosaicism was found in four mothers (7%). Three grandmothers were heterozygous for the family mutation.

Genotype-phenotype correlation

Nine patients had a missense mutation with residual creatine transporter activity. Four had mild ID and five moderate ID. IQ scores were relatively high ($n=6$, 55 ± 19) compared to the rest of the cohort (35 ± 15) although this was not significant ($p=0.055$). Six patients (aged 7-22 years) spoke in sentences and three (aged 3-16 years) spoke single words. All patients had behavioral problems but they were mild in three. Two patients had seizures, one with severe refractory epilepsy.¹⁷

The urinary Cr/Crn ratio in these nine patients was significantly lower compared to the remainder of the cohort (2.0 ± 1.0 vs 3.3 ± 1.3 ; $p=0.005$) while age at urinary sampling between these two groups was not significantly different. The creatine uptake in fibroblasts was significantly higher in 4 of the 5 patients of whom fibroblasts were available compared to the rest of the cohort ($9.3\pm1.4\%$ vs $3.1\pm2.9\%$; $p=0.0001$) but one patient (c.1190C>T; p.(Pro397Leu)) had almost no uptake (1%).

The patient with exons 5-13 deletion had a severe phenotype with severe failure to thrive, severe motor delay, profound hypotonia, dystonia, and choreo-athetoid movements.¹² The patient with exons 5-12 deletion had a moderate phenotype without movement disorder. Urinary Cr/Crn ratio was 3.8 and 1.8 respectively.

Urinary Cr/Crn ratio and creatine uptake in fibroblast did not differ significantly between patients with truncating or non-truncating mutations.

DISCUSSION

Phenotype

This survey of 101 male patients with XL-CRTR-D confirms that the most common clinical features are ID with prominent speech delay, behavioral abnormalities, and seizures, but also allows new and more detailed conclusions regarding the phenotype. Female heterozygotes were not included in this study as the phenotype in females is largely dependent on the individual X-inactivation pattern causing a wide variation in phenotype.^{25, 45}

The degree of ID varied from mild to severe with IQ scores usually between 25 and 60. It should be noted that the IQ scores are based on a limited number of patients and are probably biased towards the higher functioning patients as assessment of severely affected patients is often not initiated. Mild ID was mainly restricted to younger patients with the exception of one adult with a missense mutation with residual transporter activity.²⁰ Most adults had severe ID suggesting that patients generally develop moderate to severe ID.

However, mildly affected patients might remain more often undiagnosed.

The more pronounced ID in adults is probably due to the increasing gap between chronological and developmental age which causes a decline in IQ scores. We previously noted a similar age-related decline in IQ subscores, without regression, in the follow-up of children with CRTR-D.⁴⁴ Profound ID with IQ scores <16 were found in four middle aged brothers (31-50 years)¹⁰ and a progressive course of ID has been suggested.^{10, 15} However, evident cognitive regression was found only in one¹⁵ of the 21 adult patients in our cohort. Repeated neuropsychological assessments should be performed in adult patients to determine whether cognitive dysfunction is a progressive process.

Patients were especially delayed in speech development while motor development was only mildly delayed. Although most patients had speech limited to single words, almost one-third of the patients >10 years spoke in sentences.

Other common symptoms were behavioral problems (85%), mainly autistic features and attention deficit and hyperactivity, and seizures (59%) which were often infrequent and easily controlled and included febrile seizures. However, status epilepticus occurred and some patients had severe refractory epilepsy.

Hypotonia, mild signs of spasticity, and coordination disturbances were quite common in our cohort. Extrapyramidal movement abnormalities e.g. dystonia and athetosis are commonly associated with GAMT deficiency⁴⁶ but occurred in 11% of the CRTR-D patients in our cohort, although mostly mild. Unspecified myopathic symptoms were noted in only a few patients.

Gastrointestinal problems occurred frequently in CRTR-D patients. Feeding difficulties, vomiting, and failure to thrive were early and sometimes first presenting symptoms. Severe constipation and ileus developed later in life as previously described.^{10, 15} This might be a consequence of smooth muscle problems or autonomic nerve dysfunction which might also affect bladder function. Bladder voiding dysfunction or instability was noticed in a few patients. Caregivers should be alert to these problems, especially in older CRTR-D patients.

The below average height and slender build of most patients might be due to gastrointestinal dysfunction but could also be a consequence of cerebral or intracellular creatine depletion. Weight gain is the only consistent side effect of creatine supplementation and most likely reflects an increase in muscular mass either due to increased muscle protein synthesis or water retention in the initial days.³⁷ Cerebral creatine might also have an effect on appetite⁴⁷ and growth hormone secretion.⁴⁸

Only four patients had (mild) cardiac abnormalities and retinal abnormalities were not found in this cohort. It is remarkable that the tissues with under physiological circumstances the highest creatine content, including skeletal muscle, heart, and retina³⁷ seem to be mostly unaffected in CRTR-D. Skeletal muscle and heart have, together with kidneys, the highest creatine transporter (CRTR) expression^{37, 49} and CRTR is also expressed in the retina.^{50, 51} There are several possible explanations. First, abnormalities might be underdiagnosed because most patients did not undergo ophthalmologic and cardiac investigations. The symptoms might also present later in life. Involvement of these organs should be evaluated in a large patient cohort and physicians should be aware of the possible involvement in CRTR-D patients. Second, it is possible that these tissues acquire creatine in a different way, possibly by endogenous creatine synthesis. This implies that creatine synthesis in the creatine-requiring tissues is more important than previously expected and that these tissues do not depend on uptake only. Indeed, AGAT and GAMT enzymes are expressed in human skeletal muscle³⁷ and heart^{52, 53} and endogenous creatine synthesis has been found in the Müller glial cells of the rat retina.⁵⁴ Creatine has been found present in skeletal muscle of two CRTR-D patients by ¹H- and ³¹P- MRS³⁶ and muscle biopsy,⁵⁵ respectively. This is in contrast with the undetectable and significantly decreased creatine in the skeletal muscle and heart respectively of the CRTR-knockout mouse.⁵⁶ This difference might be explained by species differences in tissue expression of AGAT.³⁷ CRTR-knockout mice also have significantly decreased serum creatine while plasma creatine in CRTR-D patients was normal. CRTR-knockout mice might be unable to compensate decreased reabsorption of creatine in the kidneys by increasing creatine synthesis.

The brain also synthesizes creatine⁵⁷ while uptake from the periphery at the blood-brain barrier is limited.^{57, 58} Cerebral creatine deficiency in CRTR-D has been explained by the observation that GAMT is mainly expressed in glial cells and neurons might rely on CRTR to uptake creatine.⁵⁹ Others found that AGAT and GAMT are expressed in all brain cell types but rarely co-express so that intermediate GAA must be transported between AGAT and GAMT-containing cells via CRTR to insure creatine synthesis.⁵⁷ In this model GAA accumulation would be expected in CRTR-D as in GAMT deficiency. Although slightly elevated cerebral GAA was reported in one CRTR-D patient,⁶⁰ this is usually not observed.⁴⁴ Furthermore, in this cohort we found normal or only slightly elevated GAA of 0.05-0.44 μ M in CSF while CSF GAA is strongly elevated to 14-15 μ M in GAMT deficiency.⁴³ Recent studies confirmed that neurons and astrocytes are able to

synthesize creatine but showed that their intracellular creatine levels depend *in vitro* far more on uptake than *de novo* synthesis.⁶¹

Remarkably, we found normal to elevated creatine level in CSF of CRTR-D patients. In contrast, CSF creatine levels are extremely low in patients with GAMT deficiency, consistent with severely reduced cerebral creatine levels. We hypothesize that the brain synthesizes creatine but that in CRTR-D creatine is lost in CSF due to reuptake failure and that the cerebral creatine deficiency derives from defective creatine recycling.⁶² Mouse models of neurotransmitter transporter defects confirm the importance of reuptake for maintenance of intracellular neurotransmitter stores.⁶³ This supports a role of creatine as a neuromodulator as previously suggested.³⁸

Urinary excretion of specific organic acids suggesting mitochondrial dysfunction was reported in two CRTR-D patients in this cohort. Reduction of one or more respiratory chain enzyme complex activities were reported in a CRTR-D patient with a contiguous gene deletion and in three patients with either AGAT or GAMT deficiency.^{12, 64, 65} These findings suggest that creatine deficiency might also affect mitochondrial energy metabolism.

Diagnosis

All patients had an increased urinary Cr/Crn ratio compared to age-related references values⁴³ suggesting a 100% sensitive test to detect males with CRTR-D. The ratio was also increased in all patients initially diagnosed by MRS or molecular genetic screening. However, healthy controls can have elevated Cr/Crn ratio and false positives are regularly detected.⁵ A repeat morning urine sample collected after an one-day meat and fish free diet increases the test specificity.⁵ Additional molecular genetic testing of the *SLC6A8* gene is necessary to confirm the diagnosis. The urinary Cr/Crn ratio has become a widely available screening test and is in many metabolic laboratories part of the metabolic screening in ID.

¹H-MRS is also a very sensitive screening method but unavailable in many centers and requires general anesthesia. Further biochemical or molecular genetic tests are necessary to differentiate CRTR-D from creatine synthesis defects.

With the rapid development of next generation sequencing, molecular genetic screening for CRTR-D will become more common. In case of a novel unclassified variant, the diagnosis in the patient should be confirmed by creatine uptake studies in patient fibroblasts or by proving the pathogenicity of the variant. Missense variants can be classified by creatine uptake studies in CRTR deficient fibroblasts after *in vitro* overexpression of

the mutant allele.^{40, 41} Neutral or intronic variants should be studied by bioinformatic analysis and mRNA analysis.³⁹ The recommended diagnostic workup is shown in Figure 5.

Genetics

Missense mutations and 3bp deletions are most common. Two hot spot mutations c.1222_1224del; p.(Phe408del) and c.1006_1008del; p.(Asn336del) accounted together for 14% of the families. Notably, four missense mutations, c.1271G>A; p.(Gly424Asp), c.1661C>T; p.(Pro544Leu), c.1699T>C; p.(Ser567Pro), and c.1190C>T; p.(Pro397Leu) were shown to have residual transporter activity.

Mutations occurred de novo in 30% of the index patients and the recurrence risk in further pregnancies of their mothers might be expected to be low. However, we found somatic mosaicism in a relatively high percentage (7%) of mothers including a mother with two affected sons.⁶⁶ We warn that low levels of somatic mosaicism and germline mosaicism cannot be excluded with DNA sequencing and that prenatal diagnosis in further pregnancies should always be offered.

Genotype-phenotype correlation

Missense mutations with residual transporter activity in transfection studies, present in nine patients in this cohort, might be associated with a milder and more variable phenotype. Urinary Cr/Crn ratios in these patients were significantly lower and a remarkably mild ID was found in one family in two of three affected brothers²⁰ while the third brother had a more typical moderate ID, illustrating variable presentation of the same mutation. Also, one patient with a missense mutation with residual activity had severe refractory epilepsy¹⁷ while three other patients with the same mutation did not have epilepsy. In one patient, creatine uptake in cultured skin fibroblasts was very low despite a consistent residual activity of the missense mutation in the transfection studies. The results of uptake studies in patient fibroblasts are more variable and possibly influenced by other patient factors. The results of these uptake studies should therefore not be used as predictors of residual creatine transporter activity and prognosis.

A severe phenotype has been reported in three patients with multi-exon deletions of the *SLC6A8* gene.^{12, 31} We now know that the deletion included the neighbouring *BCAP31* gene in two patients but in the third patient the breakpoint was located in the non-coding region between the *SLC6A8* and *BCAP31* gene. The severe phenotype might be caused by complete loss of the *SLC6A8* gene. However, a patient with exons 5-12 deletion and patients with frameshift, splice-error or nonsense mutations in the *SLC6A8* gene did not

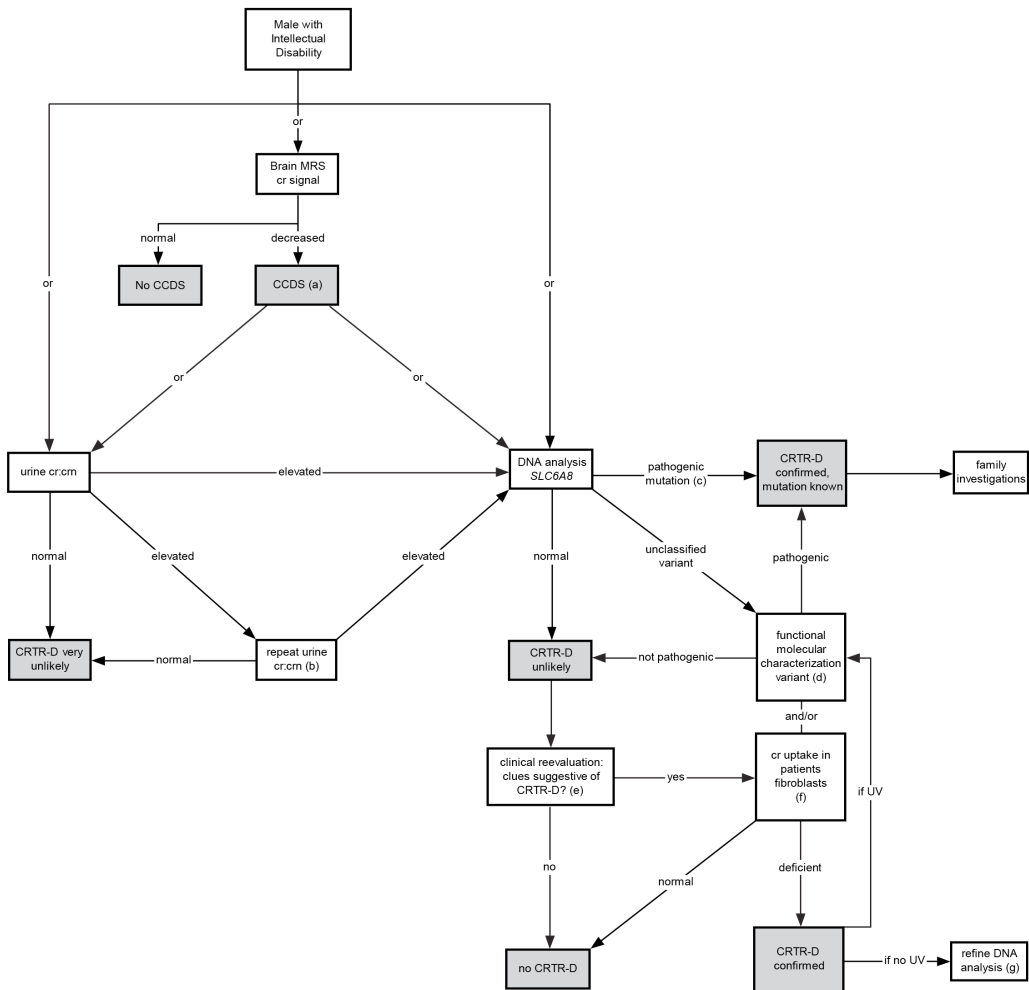


Figure 5. Diagnostic workup for CRTR-D in males. Urinary Cr/Crn ratio and creatine measured by MRS are not reliable in females and screening by DNA analysis of *SLC6A8* is recommended.⁶³ ^a Cerebral creatine deficiency syndromes (CCDS) includes AGAT-D and GAMT-D besides CRTR-D. Further evaluation should include biochemical or DNA analysis for AGAT-D and GAMT-D. ^b false positives occur, if the ratio is only mildly elevated and there is no strong suspicion of a CRTR-D, repeat the test in a morning sample after a diet devoid of meat and fish. ^c a mutation is considered to be pathogenic if it 1) is a nonsense mutation, 2) is a (3bp) deletion, 3) causes a frameshift 4) causes aberrant splicing or 5) has been proven to be pathogenic, previously. ^d If missense variant, test for restoration of creatine uptake in CRTR deficient fibroblasts after transfection with *SLC6A8* cDNA in which the variant has been introduced by site-directed mutagenesis.^{40, 41} If neutral variant or intron variance sequence (IVS), perform mRNA analysis if bioinformatic analysis points to a splice site effect.³⁹ ^e If elevated urinary cr:cm, repeat urine analysis. If repeatedly elevated urinary cr:cm, continue with brain MRS or creatine uptake in fibroblasts. If decreased brain MRS cr signal and AGAT and GAMT deficiency are excluded, continue with creatine uptake in fibroblasts. ^{d,f} If DNA analysis was the first test, perform clinical evaluation (urine Cr/Crn, possibly MRS). ^g The diagnosis is confirmed in the patient but additional molecular analysis is necessary to identify the pathogenic mutation. UV, unclassified variant.

present with the similar severe phenotype. The non-coding region between *SLC6A8* and *BCAP31* might contain a regulatory element, causing the severe phenotype in patients with deletions extending beyond the 3'end of *SLC6A8*.

Development of treatment

There is no actual treatment for CRTR-D. The effectiveness of L-arginine and glycine supplementation has not been proven.^{21, 42, 44} Cyclocreatine treatment in a brain-specific *SLC6A8* knockout mouse showed promising results⁶⁷ and warrants further studies. Awareness of the phenotype and phenotype-genotype correlation, as presented in our study, is important in the evaluation of future treatment protocols. Evaluation of the biochemical phenotype revealed high creatine in CSF suggesting a defect in reuptake of creatine in the neurons.⁶² This might direct future treatment development.

ACKNOWLEDGMENTS

This study was made possible by a grant of Metakids, obtained in 2011. The work of Ofir T Betsalel, Jiddeke van de Kamp, Grazia M S Mancini, Cornelis Jakobs and Gajja S Salomons is supported by the Metakids organization.

SUPPLEMENTARY FILES

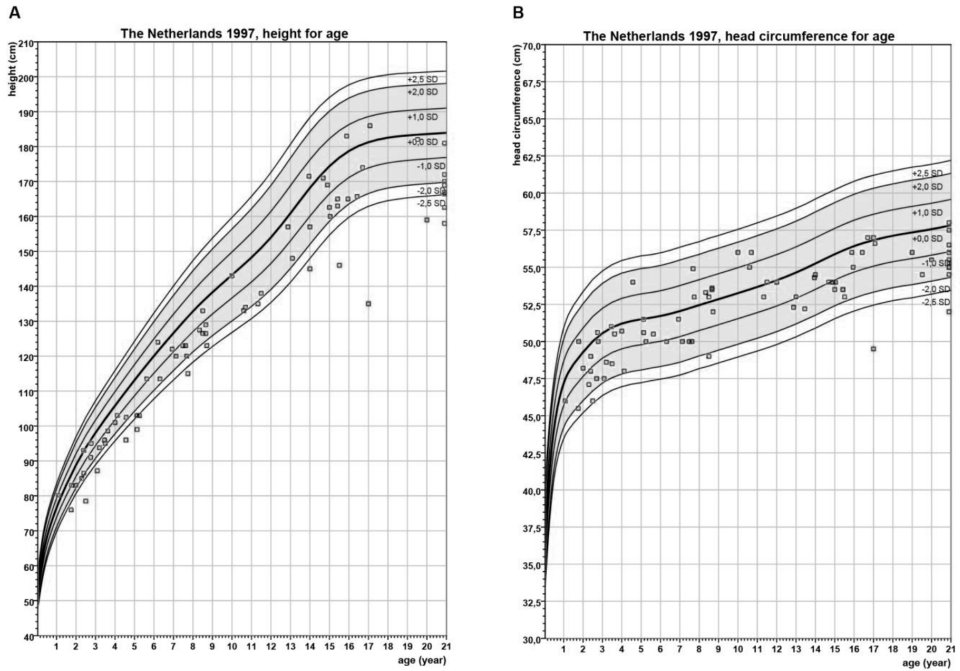


Figure 1. (a) height (n=70) and (b) head circumference (n=75) compared to Dutch reference 1997. Patients of 21 years and older are depicted at 21 years.

Table 1. Mutations detected in cohort of 103 patients from 87 families.

cDNA	Protein	Exon	Mutation type	Functional study	Families	Patients	Previously reported
c.1A>G	p.(Met1?)	1	Start codon		1	1	
c.92delC	p.(Pro31ArgfsX66)	1	Frameshift		1	2	
c.185delCAT	p.(Ile62del)	1	3 bp del		1	1	
c.219delC	p.(Asn74ThrfsX23)	1	Frameshift		1	1	Dezortova 2008
c.250_255del	p.(Lys84_Asn85del)	1	6 bp del		1	1	
c.262_262+1delinsTT	p.(?)	IVS1	Splice error	Splice prediction ^d	1	1	
c.263-2A>G	p.Gly88_Leu108del	IVS1	Splice error	Splice prediction, RNA ^d	1	1	Schiaffino 2005, Chilosi 2012
c.321_323delCTT	p.(Phe107del)	2	3 bp del	No activity ^a	2	3	Salomons 2003, deGrauw 2002
c.338G>A	p.(Gly113Asp)	2	Missense	No activity ^c	1	1	
c.370T>C	p.(Trp124Arg)	2	Missense	No activity ^b	1	1	
c.395G>T	p.(Gly132Val)	3	Missense	No activity ^b	1	1	Lion-Francois 2006
c.428_430delACT	p.(Tyr143del)	3	3 bp del		1	2	
c.462G>A	p.(Trp154X)	3	Nonsense		2	2	Fons 2009
c.541T>C	p.(Cys181Arg)	3	Missense	No activity ^b	1	1	Valayannopoulos 2012
c.570_571delTG	p.(Ala191GlnfsX10)	3	Frameshift		1	1	
c.634G>A	p.(Glu212Lys)	3	Missense	No activity ^b	1	1	
c.644+5G>A	p.(?)	IVS3	Splice error	Splice prediction ^c	1	1	
c.676G>T	p.(Glu226X)	4	Nonsense		1	1	
c.757G>C	p.(Gly253Arg)	4	Missense	Not tested	1	1	Battini 2011, Chilosi 2012
c.786 C>G	p.(Tyr262X)	5	Nonsense		1	1	Salomons 2003, deGrauw 2002
c.878_879delTC	p.(Lys293fsX3)	5	Frameshift		1	1	Poo-Arguelles 2006, Fons 2009, Fons 2010, Fons 2008
c.884_885delCT	p.(Pro295ArgfsX169)	5	Frameshift		1	1	
c.912G>A	p.Ile260_Gln304del	IVS5	Splice error	Splice prediction, RNA ^d	1	1	Lion-Francois 2006
c.917G>A	p.(Trp306X)	6	Nonsense		1	2	
c.942_944delCTT	p.(Phe315del)	6	3 bp del		1	1	Fons 2009, Fons 2010, Fons 2008
c.974_975delCA	p.(Thr325SerfsX139)	6	Frameshift		1	2	Ardon 2010
c.986G>T	p.(Ser329Ile)	6	Missense	No activity ^b	1	1	
c.993C>G	p.(Asn331Lys)	6	Missense	Not tested	1	1	Mencarelli 2011
c.1006_1008delAAC	p.(Asn336del)	6	3 bp del	No activity ^a	5	5	Clark 2006, Battini 2007, Chilosi 2008, Chilosi 2012
c.1011C>G	p.(Cys337Trp)	6	Missense	No activity ^a	1	1	Kleefstra 2005

cDNA	Protein	Exon	Mutation type	Functional study	Families	Patients	Previously reported
c.1016+2C>T	r.1017_1046del; p.(?)	IVS6	Splice error	Splice prediction ^d	2	2	Clark 2006
c.1040_1042delTCA	p.(Ile347del)	7	3 bp del		1	1	Clark 2007
c.1059_1061delCTT	p.(Phe354del)	7	3 bp del		1	2	
c.1078_1080del	p.(Phe360del)	7	3 bp del		1	2	Fons 2009, Sempere 2009
c.1090G>T	p.(Gly364Cys)	7	Missense	No activity ^b	1	1	
c.1105G>A	p.(Glu369Lys)	7	Missense	No activity ^b	1	1	
c.1141G>C	p.Gly381Arg, Val377GlyfsX15	8	Missense, splice error	No activity ^b	1	5	Hahn 2002, Salomons 2003
c.1166A>G	p.(Tyr389Cys)	8	Missense	No activity ^b	1	1	
c.1169C>T	p.(Pro390Leu)	8	Missense	No activity ^a	1	1	
c.1171C>T	p.(Arg391Trp)	8	Missense	No activity ^a	2	2	Clark 2006, Mencarelli 2011
c.1190C>T	p.(Pro397Leu)	8	Missense	Residual activity ^c	1	1	
c.1206G>A	p.(Trp402X)	8	Nonsense		1	1	
c.1210G>C	p.(Ala404Pro)	8	Missense	Not tested	1	1	Alcaide 2010
c.1222_1224delTTC	p.(Phe408del)	8	3 bp del		7	7	Poo-Arguelles 2006, Fons 2008, Fons 2009, Fons 2010, Bizzi 2002, Salomons 2003, deGrauw 2002, Valayannopoulos 2012
c.1271G>A	p.(Gly424Asp)	9	Missense	Residual activity ^b	1	3	Puusepp 2008
c.1299_1309delCCCCGGCCTCCT	p.(Pro434LeufsX27)	9	Frameshift		1	1	
c.1318dupC	p.(Arg440ProfsX25)	9	Frameshift		1	1	
c.1372_1375delGATC	p.Asp458SerfsX4	9	Frameshift		1	1	
c.1392+24_1393-30del	p.[1392_1393ins1392+1_1393-1; 1392+24_1393-30del, 1393_1495del]	IVS9	Splice error	Splice prediction, RNA ^d	2	2	Hathaway 2010
c.1417_1420delTTTG	p.(Phe473ThrfsX37)	10	Frameshift		1	1	Lion-Francois 2006
c.1428C>G	p.(Tyr476X)	10	Nonsense		1	1	
c.1456C>T	p.(Gln486X)	10	Nonsense		1	2	Garcia 2012
c.1472G>A	p.(Cys491Tyr)	10	Missense	No activity ^b	1	1	
c.1473C>G	p.(Cys491Trp)	10	Missense	No activity ^b	1	1	Lion-Francois 2006
c.1495+5G>C	p.Gly465GlufsX12	IVS10	Splice error	Splice prediction, RNA ^d	1	2	Mancini 2005
c.1519_1543delATTGCCGTATGATCGGTACCGAC	p.(Ile507LeufsX5)	11	Frameshift		3	3	Valayannopoulos 2012

Table 1. Continued.

cDNA	Protein	Exon	Mutation type	Functional study	Families	Patients	Previously reported
c.1540C>T	p.(Arg514X)	11	Nonsense		2	2	Salomons 2001, deGrauw 2002, deGrauw 2003, Salomons 2003
c.1631C>T	p.(Pro544Leu)	12	Missense	Residual activity ^b	3	4	Mancini 2005, Fons 2009, Fons 2010, Fons 2008, Mancardi 2007, Chilosi 2012
c.1661C>T	p.(Pro554Leu)	12	Missense	No activity ^a	1	1	
c.1684dupT	p.(Trp562LeufsX28)	12	Frameshift		1	1	
c.1685G>A	p.(Trp526X)	12	Nonsense		1	1	
c.1690_1703del	p.(Phe564HisfsX21)	12	Frameshift		1	1	Anselm 2008
c.1699T>C	p.(Ser567Pro)	12	Missense	Residual activity ^b	1	1	
c.778-300_1764del (2146bp)	del exon 5-12	5-12	Large deletion		1	1	
	del exon 8-13	8-13	Large deletion		1	1	Salomons 2003, Anselm 2006 ^e
	del exon 1-13 + PNCK + exon 5-8 BCAP31 ^f	1-13	Large deletion		1	1	Anselm 2006 ^e
	del exon 5-13 + exon 5-8 BCAP31 ^f	5-13	Large deletion		1	1	Osaka 2012
total					87	103	

Functional studies are mentioned for missense mutations (test for restoration of creatine uptake in CRTR deficient fibroblasts after site-directed mutagenesis) and splice error mutations (bioinformatic splice prediction and/or RNA analysis). Also mentioned are the number of patients and families with the mutation in this cohort and references to previous clinical reports of included patients.

^a Rosenberg 2007; ^b Betsalel 2012; ^c unpublished data VUmc; ^d Betsalel 2011; ^e identification breakpoints in this study; ^f further excluded from our study.

REFERENCES

1. Cecil KM, Salomons GS, Ball WSJ, Wong B, Chuck G, Verhoeven NM, Jakobs C, DeGrauw TJ. Irreversible brain creatine deficiency with elevated serum and urine creatine: a creatine transporter defect? *Ann Neurol* 2001 Mar;49(3):401-4.
2. Salomons GS, van Dooren SJ, Verhoeven NM, Cecil KM, Ball WS, DeGrauw TJ, Jakobs C. X-linked creatine-transporter gene (SLC6A8) defect: a new creatine-deficiency syndrome. *Am J Hum Genet* 2001 Jun;68(6):1497-500.
3. Rosenberg EH, Almeida LS, Kleefstra T, deGrauw RS, Yntema HG, Bahi N, Moraine C, Ropers HH, Fryns JP, Degrauw TJ, Jakobs C, Salomons GS. High prevalence of SLC6A8 deficiency in X-linked mental retardation. *Am J Hum Genet* 2004 Jul;75(1):97-105.
4. Newmeyer A, Cecil KM, Schapiro M, Clark JF, Degrauw TJ. Incidence of brain creatine transporter deficiency in males with developmental delay referred for brain magnetic resonance imaging. *J Dev Behav Pediatr* 2005 Aug;26(4):276-82.
5. Arias A, Corbella M, Fons C, Sempere A, Garcia-Villoria J, Ormazabal A, Poo P, Pineda M, Vilaseca MA, Campistol J, Briones P, Pampols T, Salomons GS, Ribes A, Artuch R. Creatine transporter deficiency: prevalence among patients with mental retardation and pitfalls in metabolite screening. *Clin Biochem* 2007 Nov;40(16-17):1328-31.
6. Clark AJ, Rosenberg EH, Almeida LS, Wood TC, Jakobs C, Stevenson RE, Schwartz CE, Salomons GS. X-linked creatine transporter (SLC6A8) mutations in about 1% of males with mental retardation of unknown etiology. *Hum Genet* 2006 Jul;119(6):604-10.
7. Lion-Francois L, Cheillan D, Pitelet G, Acquaviva-Bourdain C, Bussy G, Cotton F, Guibaud L, Gerard D, Rivier C, Vianey-Saban C, Jakobs C, Salomons GS, des Portes V. High frequency of creatine deficiency syndromes in patients with unexplained mental retardation. *Neurology* 2006 Nov 14;67(9):1713-4.
8. DeGrauw TJ, Salomons GS, Cecil KM, Chuck G, Newmeyer A, Schapiro MB, Jakobs C. Congenital creatine transporter deficiency. *Neuropediatrics* 2002 Oct;33(5):232-8.
9. Bizzi A, Bugiani M, Salomons GS, Hunneman DH, Moroni I, Estienne M, Danesi U, Jakobs C, Uziel G. X-linked creatine deficiency syndrome: a novel mutation in creatine transporter gene SLC6A8. *Ann Neurol* 2002 Aug;52(2):227-31.
10. Hahn KA, Salomons GS, Tackels-Horne D, Wood TC, Taylor HA, Schroer RJ, Lubs HA, Jakobs C, Olson RL, Holden KR, Stevenson RE, Schwartz CE. X-linked mental retardation with seizures and carrier manifestations is caused by a mutation in the creatine-transporter gene (SLC6A8) located in Xq28. *Am J Hum Genet* 2002 May;70(5):1349-56.
11. Salomons GS, van Dooren SJM, Verhoeven NM, Marsden D, Schwartz C, Cecil KM, DeGrauw TJ, Jakobs C. X-linked creatine transporter defect: an overview. *J Inherit Metab Dis* 2003;26(2-3):309-18.
12. Anselm IA, Alkuraya FS, Salomons GS, Jakobs C, Fulton AB, Mazumdar M, Rivkin M, Frye R, Poussaint TY, Marsden D. X-linked creatine transporter defect: a report on two unrelated boys with a severe clinical phenotype. *J Inherit Metab Dis* 2006 Feb;29(1):214-9.
13. Mancini GMS, Catsman-Berrepoets CE, de Coe IFM, Aarsen FK, Kamphoven JHJ, Huijman JG, Duran M, van der Knaap MS, Jakobs C, Salomons GS. Two novel mutations in SLC6A8 cause creatine transporter defect and distinctive X-linked mental retardation in two unrelated Dutch families. *Am J Med Genet A* 2005 Jan 30;132A(3):288-95.

14. Schiaffino MC, Bellini C, Costabello L, Caruso U, Jakobs C, Salomons GS, Bonioli E. X-linked creatine transporter deficiency: clinical description of a patient with a novel SLC6A8 gene mutation. *Neurogenetics* 2005 Sep;6(3):165-8.
15. Kleefstra T, Rosenberg EH, Salomons GS, Stroink H, van Bokhoven H, Hamel BCJ, de Vries BBA. Progressive intestinal, neurological and psychiatric problems in two adult males with cerebral creatine deficiency caused by an SLC6A8 mutation. *Clin Genet* 2005 Oct;68(4):379-81.
16. Poo-Arguelles P, Arias A, Vilaseca MA, Ribes A, Artuch R, Sans-Fito A, Moreno A, Jakobs C, Salomons G. X-Linked creatine transporter deficiency in two patients with severe mental retardation and autism. *J Inherit Metab Dis* 2006 Feb;29(1):220-3.
17. Mancardi MM, Caruso U, Schiaffino MC, Baglietto MG, Rossi A, Battaglia FM, Salomons GS, Jakobs C, Zara F, Veneselli E, Gaggero R. Severe epilepsy in X-linked creatine transporter defect (CRTR-D). *Epilepsia* 2007 Jun;48(6):1211-3.
18. Battini R, Chilosi A, Mei D, Casarano M, Alessandri MG, Leuzzi V, Ferretti G, Tosetti M, Bianchi MC, Cioni G. Mental retardation and verbal dyspraxia in a new patient with de novo creatine transporter (SLC6A8) mutation. *Am J Med Genet A* 2007 Aug 1;143A(15):1771-4.
19. Dezortova M, Jiru F, Petrasek J, Malinova V, Zeman J, Jirsa M, Hajek M. IH MR spectroscopy as a diagnostic tool for cerebral creatine deficiency. *MAGMA* 2008 Sep;21(5):327-32.
20. Puusepp H, Kall K, Salomons G, Talvik I, Mannamaa M, Rein R, Jakobs C, Ounap K. The screening of SLC6A8 deficiency among Estonian families with X-linked mental retardation. *J Inherit Metab Dis Online* 2009 Jan 10 doi:<http://link.springer.com/article/10.1007/s10545-008-1063-y>
21. Fons C, Sempere A, Arias A, Lopez-Sala A, Poo P, Pineda M, Mas A, Vilaseca MA, Salomons GS, Ribes A, Artuch R, Campistol J. Arginine supplementation in four patients with X-linked creatine transporter defect. *J Inherit Metab Dis* 2008 Dec;31(6):724-8.
22. Fons C, Sempere A, Sanmarti FX, Arias A, Poo P, Pineda M, Ribes A, Merinero B, Vilaseca MA, Salomons GS, Artuch R, Campistol J. Epilepsy spectrum in cerebral creatine transporter deficiency. *Epilepsia* 2009 Sep;50(9):2168-70.
23. Fons C, Arias A, Sempere A, Poo P, Pineda M, Mas A, Lopez-Sala A, Garcia-Villoria J, Vilaseca MA, Ozaez L, Lluch M, Artuch R, Campistol J, Ribes A. Response to creatine analogs in fibroblasts and patients with creatine transporter deficiency. *Mol Genet Metab* 2010 Mar;99(3):296-9.
24. Sempere A, Fons C, Arias A, Rodriguez-Pombo P, Colomer R, Merinero B, Alcaide P, Capdevila A, Ribes A, Artuch R, Campistol J. Creatine transporter deficiency in two adult patients with static encephalopathy. *J Inherit Metab Dis* 2009 Mar 25;32(Suppl 1):91-6.
25. Mercimek-Mahmutoglu S, Connolly M, Poskitt K, Horvath G, Lowry N, Salomons G, Casey B, Sinclair G, Davis C, Jakobs C, Stockler-Ipsiroglu S. Treatment of intractable epilepsy in a female with SLC6A8 deficiency. *Mol Genet Metab* 2010 Aug 26;101(4):409-12.
26. Ardon O, Amat di San Filippo C, Salomons GS, Longo N. Creatine transporter deficiency in two half-brothers. *Am J Med Genet A* 2010 Aug;152A(8):1979-83.
27. Hathaway SC, Friez M, Limbo K, Parker C, Salomons GS, Vockley J, Wood T, Abdul-Rahman OA. X-linked creatine transporter deficiency presenting as a mitochondrial disorder. *J Child Neurol* 2010 Aug;25(8):1009-12.

28. Battini R, Chilosi AM, Casarano M, Moro F, Comparini A, Alessandri MG, Leuzzi V, Tosetti M, Cioni G. Language disorder with mild intellectual disability in a child affected by a novel mutation of SLC6A8 gene. *Mol Genet Metab* 2011 Feb;102(2):153-6.
29. Garcia P, Rodrigues F, Valongo C, Salomons GS, Diogo L. Phenotypic variability in a portuguese family with x-linked creatine transport deficiency. *Pediatr Neurol* 2012 Jan;46(1):39-41.
30. Mencarelli MA, Tassini M, Pollazzon M, Vivi A, Calderisi M, Falco M, Fichera M, Monti L, Buoni S, Mari F, Engelke U, Wevers RA, Hayek J, Renieri A. Creatine transporter defect diagnosed by proton NMR spectroscopy in males with intellectual disability. *Am J Med Genet A* 2011 Oct;155A(10):2446-52.
31. Osaka H, Takagi A, Tsuyusaki Y, Wada T, Iai M, Yamashita S, Shimbo H, Saito H, Salomons GS, Jakobs C, Aida N, Toshihiro S, Kuhara T, Matsumoto N. Contiguous deletion of SLC6A8 and BAP31 in a patient with severe dystonia and sensorineural deafness. *Mol Genet Metab* 2012 May;106(1):43-7.
32. Alcaide P, Rodriguez-Pombo P, Ruiz-Sala P, Ferrer I, Castro P, Ruiz Martin Y, Merinero B, Ugarte M. A new case of creatine transporter deficiency associated with mild clinical phenotype and a novel mutation in the SLC6A8 gene. *Dev Med Child Neurol* 2010 Feb;52(2):215-7.
33. Anselm IA, Coulter DL, Darras BT. Cardiac manifestations in a child with a novel mutation in creatine transporter gene SLC6A8. *Neurology* 2008 Apr 29;70(18):1642-4.
34. Chilosi A, Casarano M, Comparini A, Battaglia F, Mancardi M, Schiaffino C, Tosetti M, Leuzzi V, Battini R, Cioni G. Neuropsychological profile and clinical effects of arginine treatment in children with creatine transport deficiency. *Orphanet J Rare Dis* 2012 Jun 19;7(1):43.
35. Chilosi A, Leuzzi V, Battini R, Tosetti M, Ferretti G, Comparini A, Casarano M, Moretti E, Alessandri MG, Bianchi MC, Cioni G. Treatment with L-arginine improves neuropsychological disorders in a child with creatine transporter defect. *Neurocase* 2008;14(2):151-61.
36. Degrauw TJ, Cecil KM, Byars AW, Salomons GS, Ball WS, Jakobs C. The clinical syndrome of creatine transporter deficiency. *Mol Cell Biochem* 2003 Feb;244(1-2):45-8.
37. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol Rev* 2000 Jul;80(3):1107-213.
38. Almeida LS, Salomons GS, Hogenboom F, Jakobs C, Schoffemeer ANM. Exocytotic release of creatine in rat brain. *Synapse* 2006 Aug;60(2):118-23.
39. Betsalel OT, Rosenberg EH, Almeida LS, Kleefstra T, Schwartz CE, Valayannopoulos V, Abdul-Rahman O, Poplawski N, Vilarinho L, Wolf P, den Dunnen JT, Jakobs C, Salomons GS. Characterization of novel SLC6A8 variants with the use of splice-site analysis tools and implementation of a newly developed LOVD database. *Eur J Hum Genet* 2011 Jan;19(1):56-63.
40. Betsalel OT, Pop A, Rosenberg EH, Fernandez-Ojeda M, Jakobs C, Salomons GS. Detection of variants in SLC6A8 and functional analysis of unclassified missense variants. *Mol Genet Metab* 2012 Apr;105(4):596-601.
41. Rosenberg EH, Martinez Munoz C, Betsalel OT, van Dooren SJM, Fernandez M, Jakobs C, Degrauw TJ, Kleefstra T, Schwartz CE, Salomons GS. Functional characterization of missense variants in the creatine transporter gene (SLC6A8): improved diagnostic application. *Hum Mutat* 2007 Sep;28(9):890-6.

42. Valayannopoulos V, Boddaert N, Chabli A, Barbier V, Desguerre I, Philippe A, Afenjar A, Mazzuca M, Cheillan D, Munnich A, de Keyzer Y, Jakobs C, Salomons GS, de Lonlay P. Treatment by oral creatine, L-arginine and L-glycine in six severely affected patients with creatine transporter defect. *J Inher Metab Dis* 2012 Jan;35(1):151-7.
43. Almeida LS, Verhoeven NM, Roos B, Valongo C, Cardoso ML, Vilarinho L, Salomons GS, Jakobs C. Creatine and guanidinoacetate: diagnostic markers for inborn errors in creatine biosynthesis and transport. *Mol Genet Metab* 2004 Jul;82(3):214-9.
44. van de Kamp JM, Pouwels PJW, Aarsen FK, Ten Hoopen LW, Knol DL, de Klerk JB, de Coo IF, Huijmans JGM, Jakobs C, van der Knaap MS, Salomons GS, Mancini GMS. Long-term follow-up and treatment in nine boys with X-linked creatine transporter defect. *J Inher Metab Dis* 2012 Jan;35(1):141-9.
45. van de Kamp JM, Mancini GMS, Pouwels PJW, Betsalel OT, van Dooren SJM, de Koning I, Steenweg ME, Jakobs C, van der Knaap MS, Salomons GS. Clinical features and X-inactivation in females heterozygous for creatine transporter defect. *Clin Genet* 2011 Mar;79(3):264-72.
46. Mercimek-Mahmutoglu S, Stoeckler-Ipsiroglu S, Adami A, Appleton R, Araujo HC, Duran M, Ensenauer R, Fernandez-Alvarez E, Garcia P, Grolik C, Item CB, Leuzzi V, Marquardt I, Muhl A, Saelke-Kellermann RA, Salomons GS, Schulze A, Surtees R, van der Knaap MS, Vasconcelos R, Verhoeven NM, Vilarinho L, Wilichowski E, Jakobs C. GAMT deficiency: features, treatment, and outcome in an inborn error of creatine synthesis. *Neurology* 2006 Aug 8;67(3):480-4.
47. Galbraith RA, Furukawa M, Li M. Possible role of creatine concentrations in the brain in regulating appetite and weight. *Brain Res* 2006 Jul 26;1101(1):85-91.
48. Schedel JM, Tanaka H, Kiyonaga A, Shindo M, Schutz Y. Acute creatine loading enhances human growth hormone secretion. *J Sports Med Phys Fitness* 2000 Dec;40(4):336-42.
49. Guimbal C, Kilimann MW. A Na(+)-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression. *J Biol Chem* 1993 Apr 25;268(12):8418-21.
50. Nakashima T, Tomi M, Katayama K, Tachikawa M, Watanabe M, Terasaki T, Hosoya KI. Blood-to-retina transport of creatine via creatine transporter (CRT) at the rat inner blood-retinal barrier. *J Neurochem* 2004 Jun;89(6):1454-61.
51. Jones EM. Na(+)- and Cl(-)-dependent neurotransmitter transporters in bovine retina: identification and localization by in situ hybridization histochemistry. *Vis Neurosci* 1995 Nov;12(6):1135-42.
52. Cullen ME, Yuen AHY, Felkin LE, Smolenski RT, Hall JL, Grindle S, Miller LW, Birks EJ, Yacoub MH, Barton PJR. Myocardial expression of the arginine:glycine amidinotransferase gene is elevated in heart failure and normalized after recovery: potential implications for local creatine synthesis. *Circulation* 2006 Jul 4;114(1 Suppl):I16-I20.
53. Schmidt A, Marescau B, Boehm EA, Renema WKJ, Peco R, Das A, Steinfeld R, Chan S, Wallis J, Davidoff M, Ullrich K, Waldschutz R, Heerschap A, De Deyn PP, Neubauer S, Isbrandt D. Severely altered guanidino compound levels, disturbed body weight homeostasis and impaired fertility in a mouse model of guanidinoacetate N-methyltransferase (GAMT) deficiency. *Hum Mol Genet* 2004 May 1;13(9):905-21.
54. Nakashima T, Tomi M, Tachikawa M, Watanabe M, Terasaki T, Hosoya KI. Evidence for creatine biosynthesis in Muller glia. *Glia* 2005 Oct;52(1):47-52.

55. Pyne-Geithman GJ, Degrauw TJ, Cecil KM, Chuck G, Lyons MA, Ishida Y, Clark JF. Presence of normal creatine in the muscle of a patient with a mutation in the creatine transporter: a case study. *Mol Cell Biochem* 2004 Jul;262(1-2):35-9.
56. Skelton MR, Schaefer TL, Graham DL, Degrauw TJ, Clark JF, Williams MT, Vorhees CV. Creatine transporter (CrT; SLC6A8) knockout mice as a model of human CrT deficiency. *PLoS One* 2011;6(1):e16187.
57. Braissant O, Beard E, Torrent C, Henry H. Dissociation of AGAT, GAMT and SLC6A8 in CNS: relevance to creatine deficiency syndromes. *Neurobiol Dis* 2010 Feb;37(2):423-33.
58. Perasso L, Cupello A, Lunardi GL, Principato C, Gandolfo C, Balestrino M. Kinetics of creatine in blood and brain after intraperitoneal injection in the rat. *Brain Res* 2003 Jun 6;974(1-2):37-42.
59. Tachikawa M, Fukaya M, Terasaki T, Ohtsuki S, Watanabe M. Distinct cellular expressions of creatine synthetic enzyme GAMT and creatine kinases uCK-Mi and CK-B suggest a novel neuronal-glial relationship for brain energy homeostasis. *Eur J Neurosci* 2004 Jul;20(1):144-60.
60. Sijens PE, Verbruggen KT, Oudkerk M, van Spronsen FJ, Soorani-Lunsing RJ. IH MR spectroscopy of the brain in Cr transporter defect. *Mol Genet Metab* 2005 Nov;86(3):421-2.
61. Carducci C, Carducci C, Santagata S, Adriano E, Artioli C, Thellung S, Gatta E, Robello M, Florio T, Antonozzi I, Leuzzi V, Balestrino M. In vitro study of uptake and synthesis of creatine and its precursors by cerebellar granule cells and astrocytes. *BMC Neurosci* 2012;13:41.
62. van de Kamp JM, Jakobs C, Gibson KM, Salomons GS. New insights into creatine transporter deficiency: the importance of recycling creatine in the brain. *J Inherit Metab Dis* 2013 Jan;36(1):155-6.
63. Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. *Nat Rev Neurosci* 2003 Jan;4(1):13-25.
64. Edvardson S, Korman SH, Livne A, Shaag A, Saada A, Nalbandian R, Iouche-Arnon H, Gomori JM, Katz-Brull R. L-arginine:glycine amidinotransferase (AGAT) deficiency: clinical presentation and response to treatment in two patients with a novel mutation. *Mol Genet Metab* 2010 Oct;101(2-3):228-32.
65. Morris AAM, Appleton RE, Power B, Isherwood DM, Abernethy LJ, Taylor RW, Turnbull DM, Verhoeven NM, Salomons GS, Jakobs C. Guanidinoacetate methyltransferase deficiency masquerading as a mitochondrial encephalopathy. *J Inherit Metab Dis* 2007 Feb;30(1):100.
66. Betsalel OT, van de Kamp JM, Martinez-Munoz C, Rosenberg EH, de Brouwer APM, Pouwels PJW, van der Knaap MS, Mancini GMS, Jakobs C, Hamel BCJ, Salomons GS. Detection of low-level somatic and germline mosaicism by denaturing high-performance liquid chromatography in a EURO-MRX family with SLC6A8 deficiency. *Neurogenetics* 2008 Jul;9(3):183-90.
67. Kurosawa Y, Degrauw TJ, Lindquist DM, Blanco VM, Pyne-Geithman GJ, Daikoku T, Chambers JB, Benoit SC, Clark JF. Cyclocreatine treatment improves cognition in mice with creatine transporter deficiency. *J Clin Invest* 2012 Aug 1;122(8):2837-46.

